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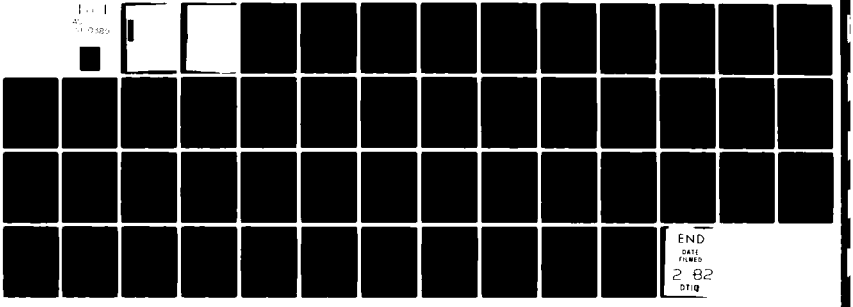

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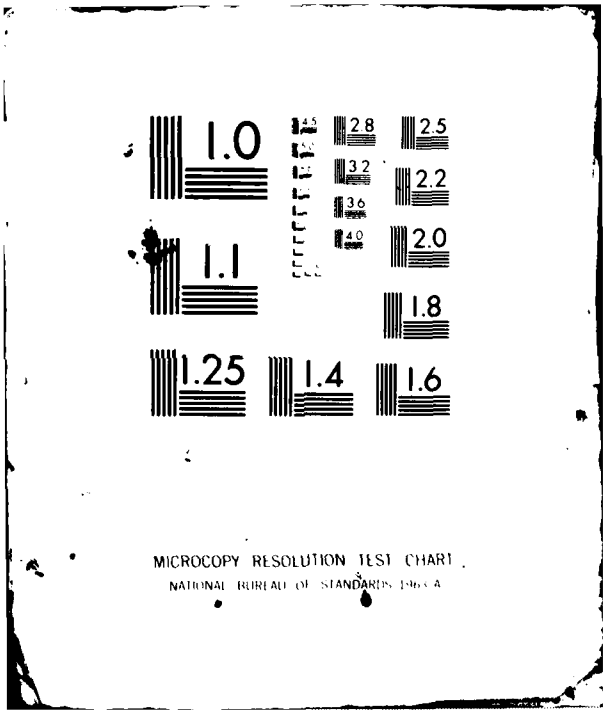
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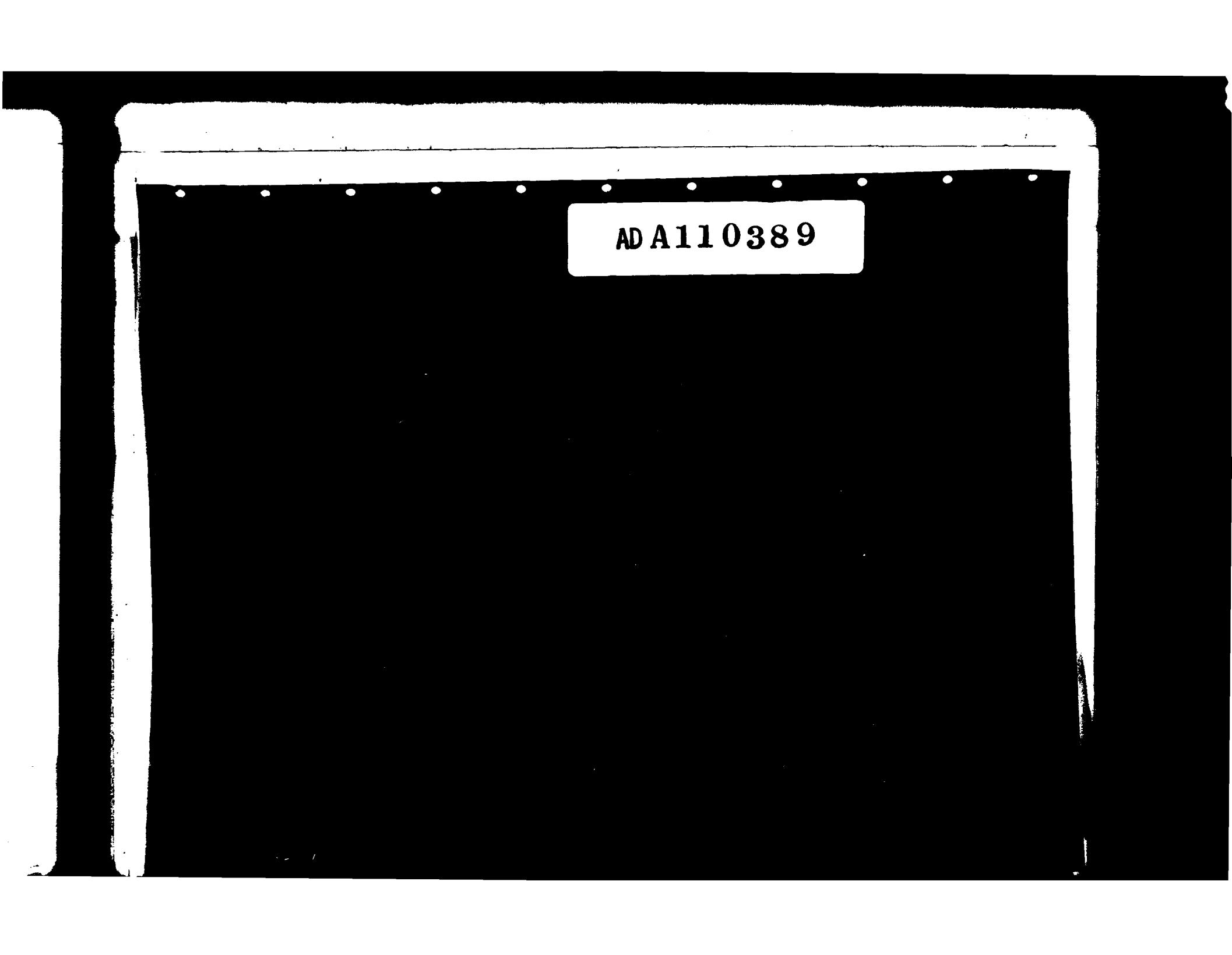

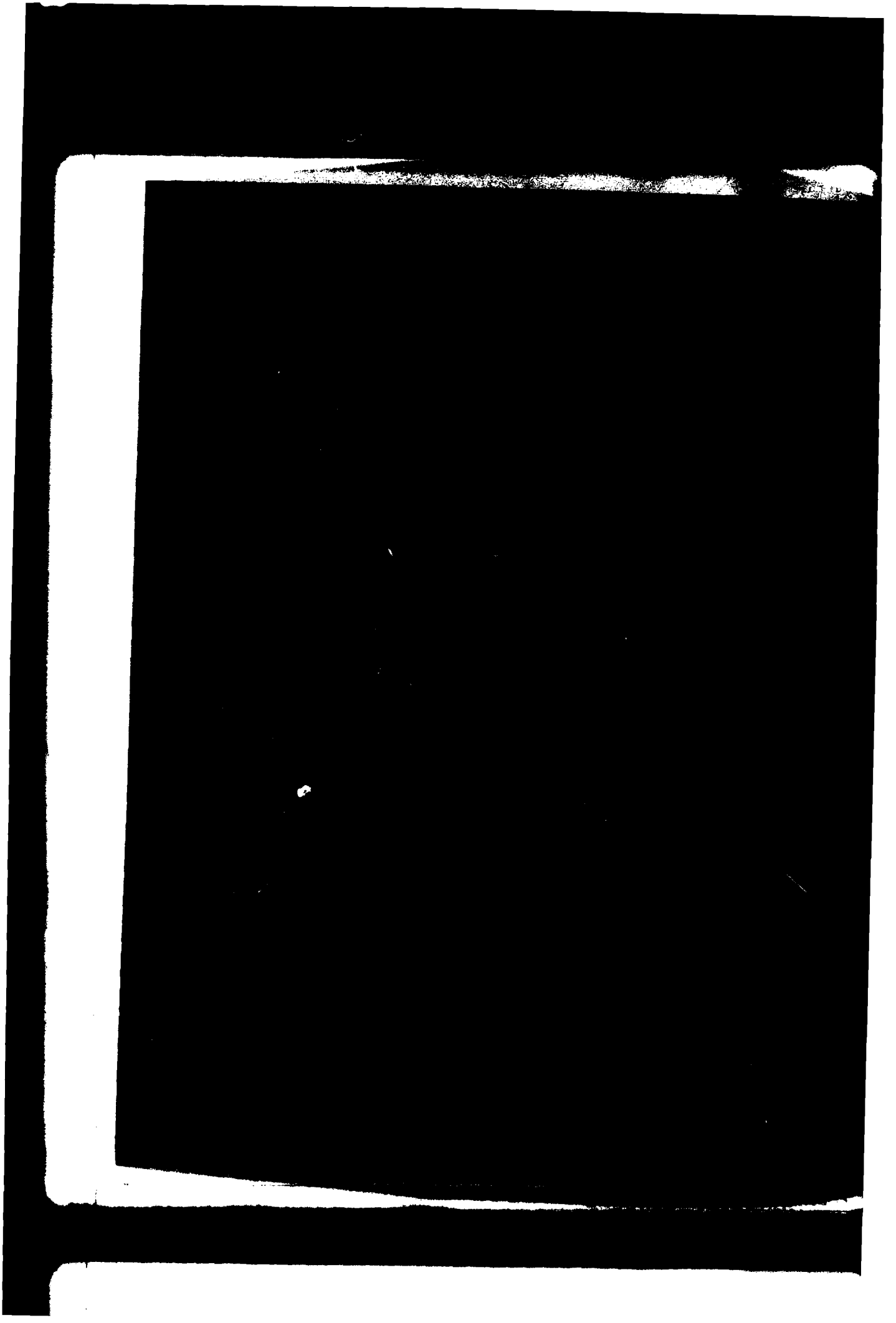

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TNT	DNT	mutant microorganisms
2,4,6-trinitrotoluene	2,4-dinitrotoluene	biodegradation
RDX	adapted microorganisms	composting
hexahydro-1,3,5-trinitro-1,3,5-triazine		sediment
		soil

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The objective of this study was to review and evaluate the available information on microbial degradation of TNT, RDX and 2,4-DNT. Particular emphasis was placed on using adapted or mutant microorganisms for degradation of low concentrations of TNT, RDX and 2,4-DNT in soils and high concentrations of these compounds in sediments. Complete mineralization of 2,4-DNT occurs rapidly in the presence of large mixed populations of microorganisms under aerobic conditions. TNT transformation also occurs under aerobic conditions, however, there is no evidence of ring

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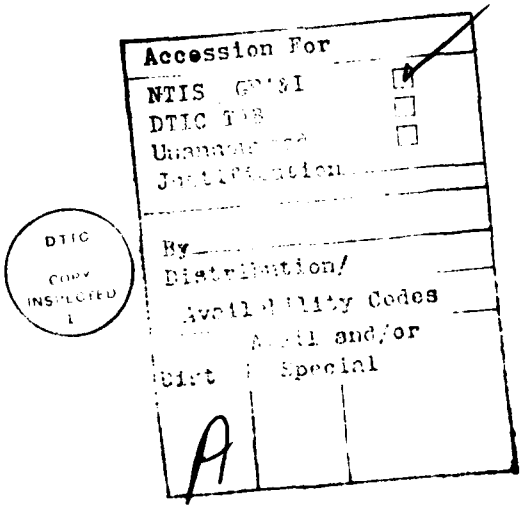
breakage. RDX degrades slowly or not at all under aerobic conditions. Anaerobic degradation of RDX yields formaldehyde and hydrazine derivatives.

Further studies into the use of adapted or mutant microorganisms for degrading TNT and RDX in soils and sediments are not recommended. Composting, however, appears to be a viable alternative for degrading high concentrations of TNT, RDX and 2,4-DNT in sediments.



SUMMARY

The objective of this study was to evaluate the effectiveness of using adapted/mutant microorganisms to degrade explosive contaminants in soils and lagoon sediments. The explosives of concern in this study were TNT (2,4,6-trinitrotoluene), DNT (2,4-dinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). To accomplish this objective, the available data on the treatment of TNT, DNT and RDX, with special emphasis on the use of adapted/mutant microorganisms, were reviewed and evaluated.

The available data indicate that 2,4-DNT undergoes transformation, not biodegradation, in the presence of pure microbial cultures and cell extracts. Degradation of the molecule occurs in the presence of mixed microbial populations with complete mineralization of 2,4-DNT within one week. In contrast, 2,6-DNT does not undergo complete mineralization.

Transformation of the TNT molecule is accomplished by most organisms (bacteria, fungi, rats, man). The 2-amino and 4-aminodinitrotoluene and condensation products account for approximately 99% of the TNT transformed in natural systems. No evidence for ring cleavage has been found in these systems. Concentration above 100 mg/l inhibit the growth of most microorganisms. TNT is transformed in enriched soils and may react with soil to form a number of products. Many of these products may be strongly sorbed to the soil and, thus, are unavailable for further degradation.

The degradation of TNT in a compost pile has also been evaluated. In contrast to the inhibition of mesophilic microorganisms at TNT concentrations above 100 mg/l, TNT at concentrations up to 10% (by dry weight) do not inhibit the composting process. The rapid transformation of TNT in this system results in soluble products with little TNT converted to CO_2 or cellular material.

RDX degradation proceeds very slowly under aerobic conditions although anaerobic degradation occurs rapidly. Several intermediates and products of anaerobic degradation of RDX have been identified including trinitrosotriazine, formaldehyde, hyrazine, symmetrical and unsymmetrical dimethylhydrazine.

All the studies on the degradation of these explosives have utilized adapted pure cultures or adapted mixed cultures of microorganisms. No biodegradation studies using mutant or genetically engineered microbes have been reported. Since 2,4-DNT degradation proceeds rapidly in the presence of mixed microbial populations, genetic engineering of microorganisms to degrade this compound is not needed. From the information available on the microbial degradation pathways of TNT, it does not appear that a microbe that would mineralize this product could be engineered. An aerobic organisms to degrade RDX could possibly be genetically engineered. However, the expense of such a project would not be warranted.

Composting appears to be the most promising method for effective, economic, ecologically acceptable disposal of TNT from lagoon sediments. Composting should also be effective for decontamination of sediments containing DNT based on structural similarity to TNT and aerobic degradation of this compound by mixed microbial populations. It is also possible that this technique may be applicable to disposal of RDX because of the aerobic/semianaerobic conditions coupled with the activity of thermophilic microorganisms found in the compost system. Further evaluation of this technique and its biodegradation products should be undertaken.

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I. INTRODUCTION

A. Objective

The objective of this study was to evaluate the applicability of utilizing adapted/mutant microorganisms for effecting the degradation of explosives in soils and sediments to environmentally acceptable products. The explosives considered in this evaluation were RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), TNT (2,4,6-trinitrotoluene) and DNT (2,4-dinitrotoluene). The concentrations of these explosives vary from the low ppm level in soils to approximately 100,000 $\mu g/g$ in wastewater lagoon sediments. Thus, the ability of the microorganisms to survive in and degrade the explosives at various concentration levels was also evaluated. The utility of adapted/mutant microorganisms for degradation of the explosives is predicated on the environmental hazards of the products. Thus, the identification of the degradation pathways, the intermediate and final products and the environmental hazards of these products was of prime concern to this study.

B. Background

RDX and TNT are the major explosives used in conventional weaponry by the United States. These explosives are manufactured by the Army at various Army Ammunition Plants located throughout the United States. The Army also performs the major portion of the shell loading operations of these explosives although the Air Force and Navy maintain some shell loading facilities. Although DNT is mainly used in propellants, it is also an intermediate in the manufacture of TNT and is often present in small quantities in the TNT final product.

As a result of the manufacture and handling of these explosives, soils around the facilities have become contaminated by low levels of these compounds due to dust emissions and spills. In general, the soil concentrations are in the low ppm range. In contrast to the low contamination found in soils, lagoon sediments contain large concentrations of these explosives, *i.e.* approximately 10%. These lagoons are used for wastewater disposal from shell loading and cleaning operations. In general, the wastewaters contain less than 100 mg/l of the explosives, however, over the years these explosives have precipitated out of the water and collected in the lagoon bottom sediments.

One potential cost-effective method for cleaning up TNT, RDX and DNT in soils and sediments is biological degradation of these explosives. This report investigates the feasibility of biological degradation as a decontamination technique for soils and sediments. The adapted or mutant strains of microorganisms are of particular interest to this study since it is in the area of adapted/mutant biodegradation that new advances in degradation of environmentally recalcitrant chemicals are being made. For definition purposes, adapted microorganisms are naturally occurring species which adapt to specific, usually undesirable, environmental conditions. Adaptation may be a gradual process with incremental increases in potentially toxic or inhibitory substances, incremental increases or decreases in temperature, or decrease of one nutrient while increasing a potential substitute. Adaptation of microorganisms to a particular environment occurs quite rapidly and results in microbial populations which have successfully altered substrate requirements, growth requirements or other metabolic functions. However, such adaptation is transient and often disappears when the environmental conditions return to the natural state.

Mutant microorganisms are those organisms which have undergone a mutagenic event with resultant alteration of the bacterial DNA. Mutant organisms occur in nature or may be created by a programmed treatment with radiation or other mutagenic agent. These organisms may acquire new characteristics such as increased or decreased permeability, increased or decreased production of specific enzymes, etc. Selection for the microorganisms with the desired characteristics can then result in large populations of mutant microbes which can degrade specific chemicals.

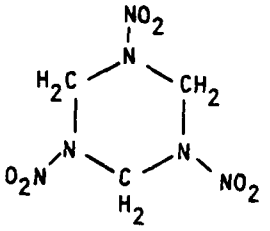
Genetic engineering utilizes a host microorganism which tolerates the environmental conditions specified (e.g. presence of TNT, DNT, RDX) and a donor microorganism which has the capability to perform a required task (e.g. enzymatic reduction, oxidation, ring cleavage) but cannot tolerate the environmental conditions specified. DNA is isolated from the donor organism, fragmented by restriction endonucleases and inserted into the DNA of a plasmid or a virus for introduction into the host microorganism. Selection of the new microorganism containing the desired genetic information is then carried out and the selected organism is cultured in large quantities.

II. REVIEW OF THE LITERATURE ON MICROBIAL DEGRADATION OF EXPLOSIVES

A. RDX (Hexahydro-1,3,5-trinitro-1,3,5-triazine)

1. Physical and Chemical Properties of RDX

RDX is a cyclic nitramine explosive having the following structure:



The solubility of RDX in water is low, *i.e.* 44.7 mg/l at 18°C (Spanggord, 1977) or 42 mg/l at 20°C (Sikka *et al.*, 1980). Spanggord *et al.*, (1980) have reviewed the literature on the environmental fate of RDX. According to the best available information, volatilization of RDX from water is not a significant factor in the environmental fate of this explosive. The estimated half-life for volatilization is 9×10^6 days (Spanggord *et al.*, 1980). RDX is only weakly adsorbed to sediments with a partition coefficient of 4.2 for high organic sediment and 0.8 for sandy-loam (Sikka *et al.*, 1980).

RDX undergoes photolysis in both sunlight (wavelength greater than 290 nm) and under shortwave ultraviolet light (wavelength = 254 nm). The major product appears to be the mononitroso analog of RDX (Kubose and Hoffsommer, 1977). Half-life estimates for photolysis of RDX in aqueous solutions range from 10.7 hours (Sikka *et al.*, 1980) to 7 days (Spanggord *et al.*, 1978). In contrast to photolysis, hydrolysis in the environment is relatively slow. The hydrolysis half-life in seawater is 1.7 years at pH approximately 8 and 25°C (Hoffsommer and Rosen, 1973). The half-life for alkaline hydrolysis, pH 9, of RDX is reported to be 200 days (Hoffsommer *et al.*, 1977).

Chemical reduction of the nitro groups is the most fascile reaction. Depending on the reducing agent and media, N-hydroxylamine, N-nitroso and N-amino compounds can be formed.

2. Microbial Degradation of RDX

Several researchers have studied the degradation of RDX by microorganisms under a variety of conditions. The results of these studies are summarized in this section.

Osmon and Klausmeier (1973) investigated microbial degradation of RDX. Organisms capable of growth in complex media saturated with RDX were readily obtained. Some RDX disappearance was indicated during soil enrichment studies, however, proof of RDX degradation by microorganisms was not obtained. No experimental data is presented in this chapter of "Developments in Industrial Microbiology" and no further investigation of the biodegradation of RDX by this group was reported.

Soli (1973) investigated the anaerobic degradation of RDX by purple photosynthetic bacteria belonging to the families *Thiorhodaceae* and *Athiodaceae*. A defined salt solution containing an organic source of carbon and nitrogen at pH 7-8 was used as a culture medium (salinity approximately 3-4 ppt). RDX was dissolved in the medium at a concentration of 40 mg/l and sterilized by filtration. The medium was mixed 1:1 with a similar sterile medium containing ammonium chloride and sodium acetate and no RDX (final concentration of RDX was 20 mg/l). The RDX medium was divided into three portions. One portion was maintained as the sterile control. A second portion was inoculated with a mixed population of photosynthetic bacteria and the third portion with *Chromatium* species. bacteria. The cultures were incubated at 27°C under continuous artificial light and strict anaerobic conditions. Disappearance of RDX was monitored by measuring UV absorbance at 240 nm. Results were confirmed by GC using an electron capture detector (^{63}Ni). After a few days days (exact time not specified), the sterile control showed no disappearance of RDX. With the mixed populations, 97% of the RDX disappeared. Sixty percent RDX disappearance was found with the *Chromatium* species. No attempt was made to identify degradation products or to determine if ring cleavage had occurred.

In a 3-year pilot plant study, Hoffsommer *et al.* (1978) monitored the degradation of TNT in the presence of RDX. The initial concentrations were 15.1 mg/l of TNT and 7.3 mg/l of RDX. With aerobic activated sludge microorganisms in a batch experiment, 99.6% of the TNT was bioconverted when present in the medium alone or in the presence of RDX. No bioconversion of RDX was observed.

Microbial degradation of RDX using water and sediment samples collected from the Holston River and wastewater effluents from the Holston Army Ammunition Plant, Kingsport, Tennessee was investigated by Sikka *et al.* (1980). Previous experiments had indicated that 10 mg/l RDX was not toxic to microorganisms in lake water, thus, these experiments were performed at an initial concentration of 10 mg/l ^{14}C-RDX. The water samples were incubated in the dark in an environmental control chamber at 18 ± 1°C (No information on aerobic/anaerobic conditions utilized was given.). The $^{14}CO_2$ resulting from degradation of ^{14}C-RDX was measured using a biometer flask with a CO_2-trapping solution in the side arm. The amount of $^{14}CO_2$ in the CO_2-trapping solution was measured on a Packard Tricarb liquid scintillation counter. Water amended with bottom sediment (1.0 g/100 ml) or an external carbon source (e.g., yeast extract, 5 mg/100 ml) was used in these studies. Degradation of RDX was also monitored in a sterile water sample to determine the extent of any non-biological degradation.

The evolution of $^{14}CO_2$ as a function of incubation time is shown in Figure 1. The rate of $^{14}CO_2$ evolution was greatest in water amended with 1% sediment obtained from an HMX production-water waste stream. No reference to HMX content in the sediment or water was noted. More than 80% of the ^{14}C added as RDX in this sample was evolved as $^{14}CO_2$ after 38 days of incubation. Water samples obtained from the HMX production-water waste stream (no sediment added) and the Holston River (amended with yeast extract) yielded 20% of the total ^{14}C added evolved as $^{14}CO_2$. Other river water samples with sediments did not produce significant amounts of $^{14}CO_2$ despite their ability to transform RDX (approximately 80% decrease from initial 10 mg/l level).

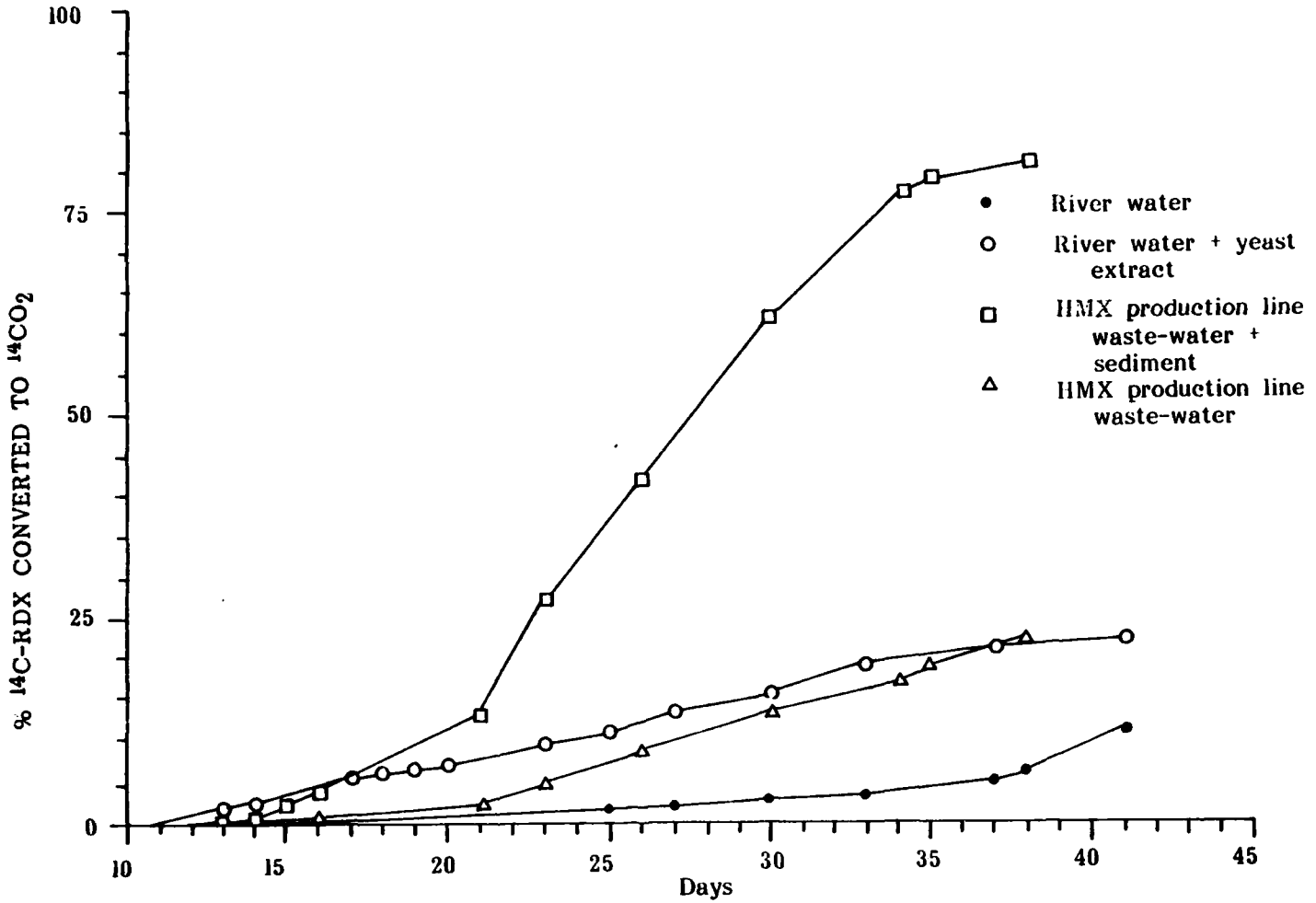


Figure 1. Evolution of $^{14}CO_2$ from water samples incubated with ^{14}C-RDX (Sikka *et al.*, 1980)

Attempts were made to isolate and identify RDX biodegradation products. Samples were first extracted with ethyl acetate at pH 7.0. The aqueous layer was then acidified to pH 2.0 and re-extracted with ethyl acetate and water saturated n-butanol in succession. The ethyl acetate extracts were concentrated under vacuum at 30°C in the dark and the radioactivity of each fraction was determined. Small quantities of RDX biodegradation products were isolated, however, no characterization or identification of these products was possible. The studies indicated that RDX is degraded by the activity of aquatic microorganisms and that this degradation is enhanced in the presence of sediment. As noted by the investigators, further studies are needed to confirm the evolution of $^{14}CO_2$ from samples containing ^{14}C-RDX.

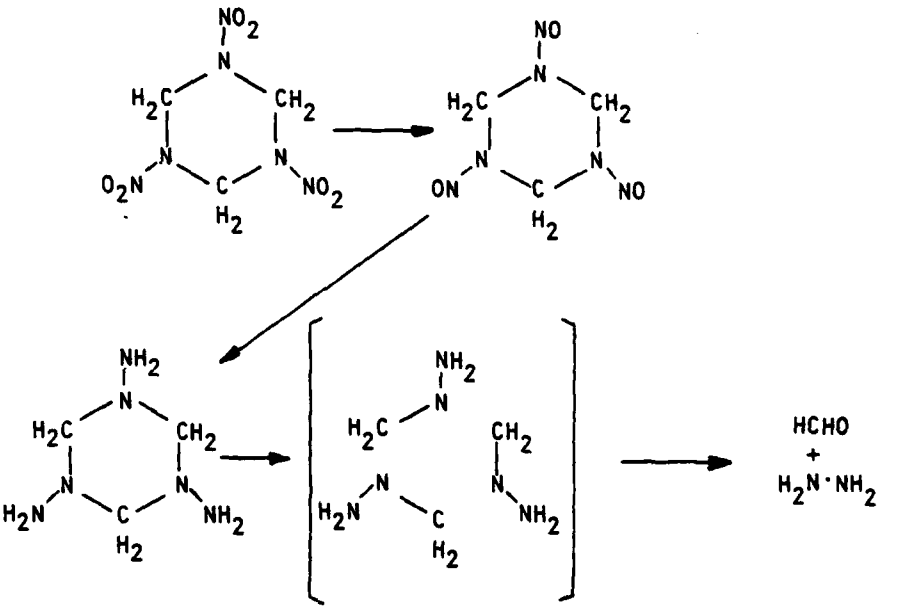
SRI (1980) reports that aerobic biodegradation of RDX occurs only in the presence of sediments and occurs slowly following a lag phase. Under anaerobic conditions, RDX is degraded in a few days in the presence of yeast extract. Formaldehyde was tentatively identified as one of the biodegradation products. No additional data are available at this time.

Biodegradation studies of ^{14}C-labeled RDX were reported by Natick (1980). No degradation was observed under aerobic conditions. Rapid disappearance of ^{14}C-RDX was observed from static nutrient broth cultures and from chemostat studies utilizing a medium high in nitrates, low in phosphates and amended with beet sugar molasses. These cultures were inoculated with anaerobic sludge microorganisms which were acclimated to RDX at less than 1 μg/ml (Natick, 1980). Experiments were conducted at temperatures ranging from 30° to 37°C and at RDX concentrations of 10 to 50 mg/l. Throughout the experiments, the supernatant fraction contained 98-99% of the radioactivity from ^{14}C-RDX. To recover any ^{14}C gases, the cultures were purged with helium through a series of traps. Recovery of ^{14}C from the traps was 1.5% of the initial label while 98.5% remained in the aqueous fraction. Concentration of the aqueous phase resulted in loss of radioactivity, suggesting the formation of labile, polar ^{14}C-compounds. Distillation of the reaction mixture yielded a distillate containing 20% of the label. This distillate gave a positive chromotropic acid test for formaldehyde. The reaction of the distillate with methone yielded yellow-white crystals with a melting point of 191°C (M.P. of the formaldehyde derivative of methone is 189°C). The crystalline derivative was radioactive. The formaldehyde concentration peaks in a few days with complete disappearance of the formaldehyde within three weeks. Dr. McCormick (1980) indicated that the formaldehyde appeared to be reduced to methanol.

Chloroform extraction of the reaction mixture yielded yellow crystals upon crystallization from ethanol. The compound was identified by TLC, GC, GC/MS and IR as trinitrosotriazine, a reduction product of RDX. The crystals had high specific activity of ^{14}C. No attempt was made to quantitate the yield. Trinitrosotriazine was identified as a product in static cultures only; it is not clear if trinitrosotriazine is an intermediate in formaldehyde formation. A subsequent report states that an acid-stable, alkaline-volatile material present in reaction mixtures formed a crystalline derivative with salicyaldehyde which was identical to authentic salicylazine on GC/MS analysis and the product was identified as hydrazine. MS analysis indicated the presence of hydrazine as well as symmetric and asymmetric dimethylhydrazine.

The presence of RDX in static cultures was monitored by HPLC and showed a reduction in RDX with the appearance of two new peaks after 1 day. In 2 days, the RDX had almost completely disappeared, the two new peaks had decreased and a third peak appeared. These peaks do not correspond to any of the compounds previously identified as RDX biodegradation products and studies are underway to determine the identity of the compounds.

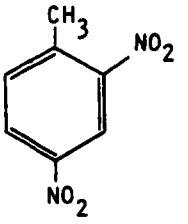
Comprehensive reports on RDX degradation have not yet been published by Dr. Kaplan and his coworkers, thus, all material presented here has been obtained from brief monthly reports and from a telephone conversation with Dr. McCormick. From the information available, it appears that RDX is rapidly degraded by an anaerobic sludge microbial population in concentrations up to 50 mg/l (above or near the maximum solubility of RDX in aqueous solutions). The proposed pathway for the breakdown of RDX by anaerobic microorganisms is shown below (Natick, 1979).



B. DNT (2,4-Dinitrotoluene)

1. Physical and Chemical Properties of DNT

DNT (2,4-Dinitrotoluene) is as nitroaromatic having the following structural formula:



The solubility of DNT in water is reported to be 27 mg/100 g (Desvergnes, 1925). Spanggord *et al.* (1980) have recently reviewed the available information on the environmental fate of DNT. No data on the rate of volatilization of DNT from water, sorption onto soil or hydrolysis were found. DNT is photolyzed under both shortwave ultraviolet and sunlight. Photolysis occurs rapidly at wavelengths greater than 290 nm. The photolysis rate is pH dependent and occurs more rapidly in basic solution (pH 10.8) than in acid medium (pH 3.3). Degradation products identified resulted from reduction of one or both nitro groups and oxidation of the methyl group.

2. Microbial Degradation of DNT

In early studies, Cartwright and Cain (1959) reported non-specific nitroreductase activity in extracts of *Nocardia erythropolis* resulting in the conversion of dinitrobenzoic acid to the corresponding amino form. Chambers *et al.* (1963) investigated the degradation of aromatic compounds by phenol-adapted bacteria. *Psuedomonad* sp. were the predominant microorganisms. These investigators reported evidence of some oxidation of 2,4-DNT in the presence of the phenol-adapted bacteria. Adapted microorganisms were grown in mineral salts medium with vitamin B_{12} added. The only source of carbon was DNT supplied at 100 mg/l. Respirometric techniques were used at a test temperature of 30°C and observations were made at 10 minute intervals. Steady but slow oxygen uptakes were observed in cultures with 2,4-DNT and 2,6-DNT, however, the author observed that the limited activity detected under laboratory conditions may bear little relationship to activity in a practical waste treatment operation.

Villaneuva (1961) isolated a strain of *Nocardia* (*Nocardia* v) which reduced a variety of nitro compounds to the correspondng amino derivatives. Nitro compounds (30 μ/ml) were added to a system consisting of l-cysteine ($1x10^{-4}$M), *l*-malic acid ($1x10^{-2}$M) in a 0.067 M phosphate buffer, pH 7.6. A 2.5 ml suspension of early stationary phase organisms grown on glutamic acid medium was added to make a final concentration of 5 mg/ml. After 1 hour incubation at 37°C, 0.1 ml of 40% trichloroacetic acid was added and the mixture centrifuged. Aliquots from the supernatant were tested spectrophotometrically for arylamines.

McCormick *et al.* (1976) reported the reduction of 2,4-DNT in a system utilizing cell free extracts of the strict anaerobe *Veillonella alkalescens.* The cell free extracts catalyzed the reduction of the nitro groups of DNT by hydrogen gas. Both nitro groups on the DNT molecule can be reduced, however, rate studies suggest that the reduction of 2,4-DNT proceeds with reduction of the 4-nitro group first. After 10 minutes, 4-amino-2-nitrotoluene was identified in the mixture. After 60 minutes, only 2,4-diaminotoluene was found in the solution.

Mucrosporium sp. were incubated in a synthetic medium containing 1% glucose and DNT (100 mg/l) with shaking at 30°C (McCormick *et al.*, 1978). Samples were removed for analysis at various intervals after the addition of DNT. The mycelia were recovered by filtration through glass wool and membrane filters. The supernatant was extracted three times with dichloromethane, the extract evaporated to a small volume and subjected to TLC. UV quenching areas were scraped off and extracted with dichloromethane. Identities of unknown products were confirmed by GC/MS. Products identified were 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, 2,2'-dinitro-4'-azoxytoluene, 4,4'-dinitro-2,2'-azoxytoluene and 4-acetamido-2-nitrotoluene. A third azoxy compound believed to be a "mixed" type (i.e., 2,4'-azoxy or 4,2'-azoxy) was isolated but not identified. The proposed biodegradation pathways for DNT are shown in Figure 2.

Parrish (1977) screened 190 fungi representing 98 genera for their ability to transform 2,4-dinitrotoluene. Fungi were grown in shake cultures at 29°C in basal medium with 0.5% glucose. DNT was added at 0 to 5 days after inoculation of the culture medium to an initial concentration of 100 mg/l. Culture filtrates were analyzed by liquid chromatography. Only five organisms were able to transform DNT. Transformation was preceded by an induction period of about 8 hours and was complete in approximately 24 hours. Transformation products were the same as those obtained by McCormick *et al.* (1978) and no evidence for cleavage of the carbon skeleton was observed.

The Stanford Research Institute (1980) reports rapid biodegradation of 100 mg/l DNT by high concentrations of mixed microbial populations with complete mineralization of the carbon. DNT was found to have a half-life of 1-2 days with total degradation achieved within one week. $^{14}CO_2$ evolved in 1-2 days was equal to 60% of the ^{14}C-label added as DNT. The formation of amino intermediates and some condensation products was observed. Only very small quantities of azoxy polymers were found. Mixed population microbial degradation of ^{14}C-2,6-DNT yielded no $^{14}CO_2$.

Transformation of DNT has been demonstrated in both pure culture and cell extract. However, rapid biodegradation of DNT with complete mineralization occurs only in the presence of high concentrations of mixed microbial populations. Mineralization of 2,6-DNT does not occur in the presence of mixed microbial cultures.

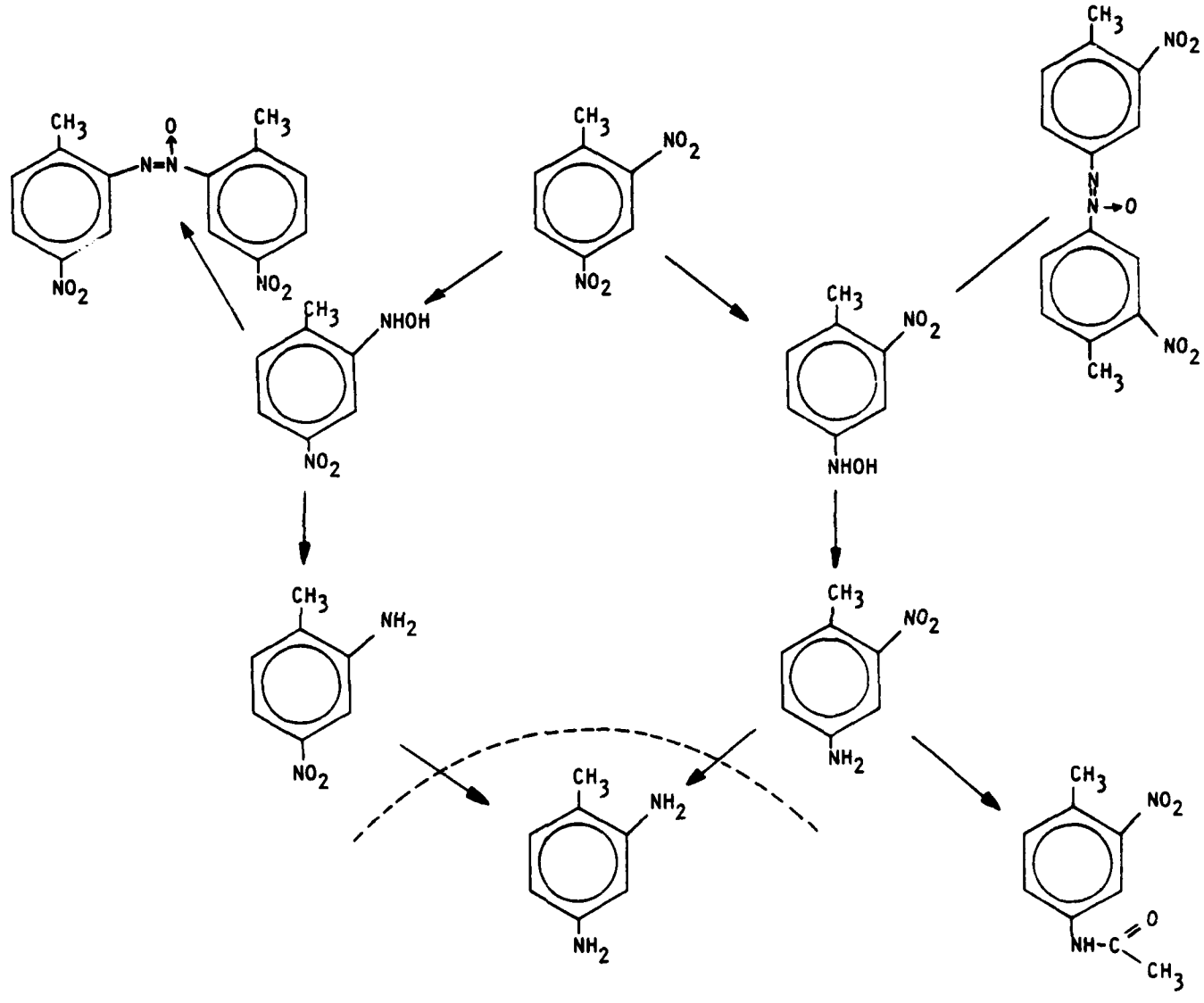
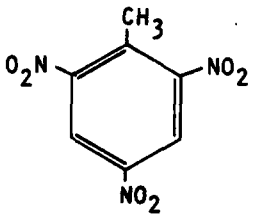


Figure 2. Scheme for pathway for formation of transformation products from 2,4-Dinitrotoluene (McCormick *et al.*, 1978)

C. TNT (2,4,6-Trinitrotoluene)

1. Physical and Chemical Properties of TNT

2,4,6-Trinitrotoluene (TNT) is a high explosive having the following structural formula:



The water solubility of this compound has been measured to be 0.013 g/100 g (Urbanski, 1964) or 117 mg/l (Spanggord, 1977). These two values are in good agreement with the average value of 124 mg/l. Spanggord *et al.*, (1980) estimated the half-life for volatilization of TNT from water to be 990 days. Sorption of the TNT itself on soil does not appear to play a major role in the environmental fate of the compound. Sikka *et al.* (1980) measured the partition coefficient, K_p, of TNT in four sediments after 24 hour equilibrium times. The K_p values measured ranged from 5.5 to 19.3. Spanggord *et al.* (1980) estimated K_p to be 13.3 using the data of Karickhoff (1978), Kanaga and Goring (1978), and Smith *et al.* (1978). Hydrolysis of TNT will also not play a significant role in the environmental fate of this compound (Spanggord *et al.*,.1980).

Photolysis is a major pathway for degradation of TNT in the environment leading to the formation of "pink water." TNT absorbs light in the region above 290 nm. The absorption coefficients of TNT in water were reported to be 650, 250 and 30 M^{-1} cm^{-1} at 300, 350, and 400 nm, respectively (Abe, 1959).

The sunlight photolysis of TNT in natural river waters and distilled water was studied by Burlinson (1978). In the natural river water (pH 8.2), TNT completely disappeared after 6 to 8 days. The main photolysis product was 1,3,5-trinitrobenzene, although 2,4,6-trinitrobenzaldehyde and aminonitro biotransformation products were also identified. In addition to these compounds, 2,4,6-trinitrobenzonitrile, 4,6-dinitroanthanil and substituted azoxybenzene compounds have been reported as photolysis products and pink water constituents (Burlinson *et al.*, 1973). In distilled water, only 2,4,6-trinitrobenzaldehyde was produced.

The photolysis of TNT at wavelengths greater than 290 nm is pH dependent. At pH 1.1, only 3% of the TNT is lost after 60 minutes while 49% and 70% TNT losses were reported at pH 3.0 and 6.0 (Burlinson *et al.*, 1973).

2. Microbial Degradation of TNT

a. Aerobic Degradation of TNT

The microbial degradation of TNT has been studied by many investigators. In general, the action of bacterial and fungal species on TNT results in a variety of reduction products including 2-amino and 4-aminodinitrotoluene and azoxy dimers. Potential biotransformation pathways for obtaining these intermediates and products are shown in Figure 3.

Enzinger (1970) reported reduction of 100 mg/l of TNT to 1 mg/l in broth cultures. Bio-oxidation units were not adversely affected by TNT at 29 mg/l. Two unidentified biodegradation compounds were reported. The compounds were probably the 4-amino and perhaps the 4,4'-azoxy compounds because GC analysis indicated that the compounds were not 2,4-DNT and 2,6-DNT.

Nay *et al.* (1974) at Virginia Polytechnical Institute studied the biodegradability and treatability of TNT manufacturing wastewater in an activated sludge system. The rate of TNT disappearance was relatively slow and it was concluded that conventional activated sludge processes were not acceptable as a method for biological treatment. A combination of biological and chemical processes was recommended to overcome the inherent difficulties of biological treatment and the expense of chemical treatment. No attempt was made to identify TNT transformation products in these studies.

Klausmeier (1973) and Osmon and Klausmeier (1973) isolated a number of bacteria and fungi that were effective in rapidly transforming TNT into the reduction products found in earlier investigations. None of the organisms tested could utilize TNT as a sole carbon source. The rate of TNT disappearance was related to the microbial activity which in turn was dependent upon an organic nutrient source.

Klausmeier and coworkers (1974) also studied the effects of TNT on a variety of microorganisms under varied conditions. TNT at concentrations greater than 50 mg/l prevented or severely inhibited growth of most fungi, actinomycetes, and Gram-positive bacteria. Most organisms grew when TNT concentrations did not exceed 20 mg/l. Many Gram-negative bacteria grew well at concentrations of 100 mg TNT/l or more. Growth of any microorganisms in media containing TNT resulted in a decrease in the level of TNT in the medium; however, no evidence of ring cleavage was found.

The fate of ^{14}C-labeled TNT in an activated sludge system was investigated by Carpenter *et al.* (1978). ^{14}C-TNT disappeared from the reactor within 3 to 5 days, however, no significant $^{14}CO_2$ was formed (less than 0.5% of radioactivity). Radioactivity was about equally divided between the floc and the supernatant. The ^{14}C in microflora was not the characteristic constituent of lipid and protein material (fatty acids and amino acids) but was bound to the compounds as polyamide macromolecules. No evidence of ring cleavage was observed.

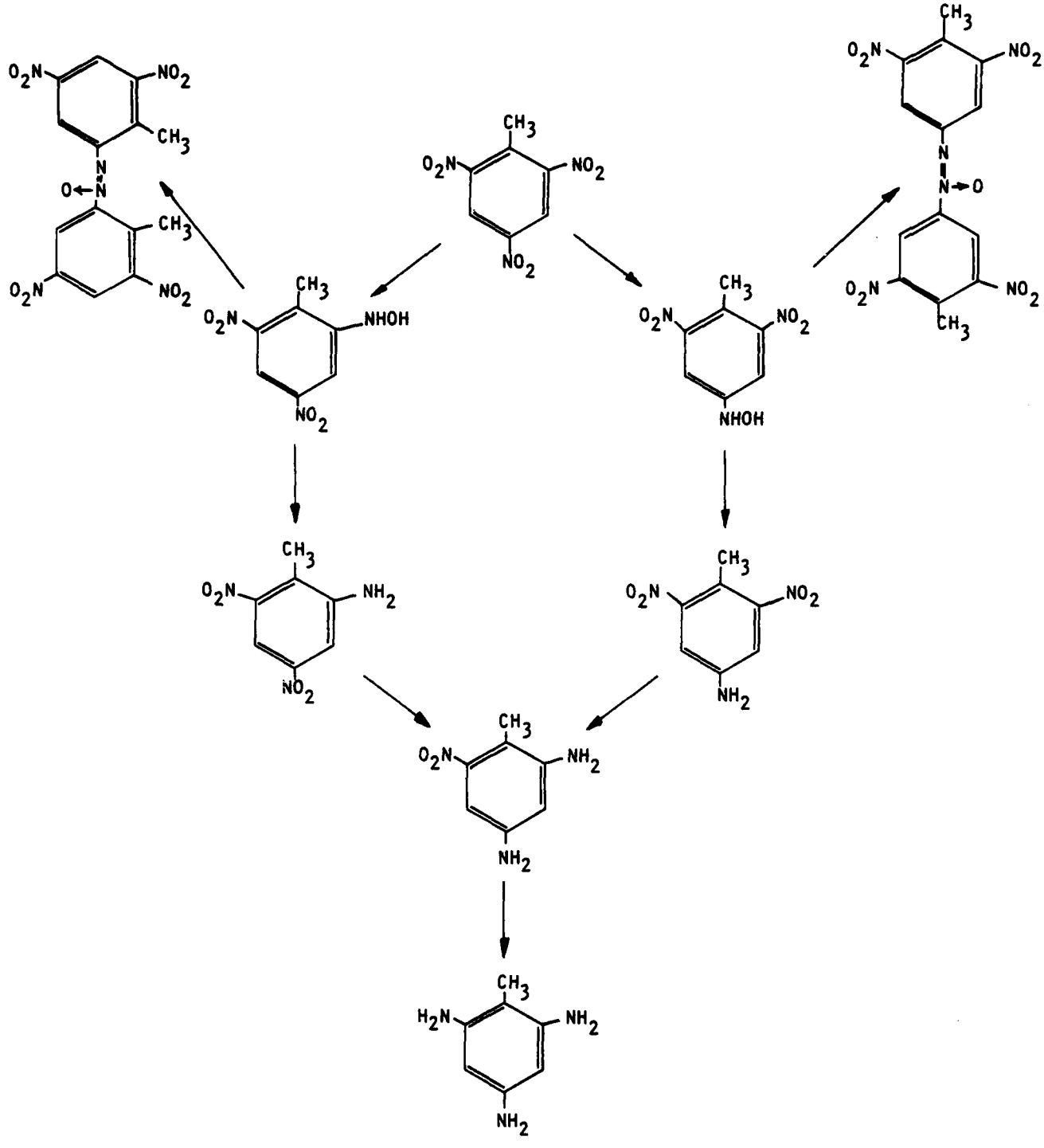


Figure 3. Scheme for pathway for formation of transformation products from 2,4,6-trinitrotoluene (McCormick *et al.*, 1976)

Hoffsommer *et al.* (1978) have completed a three-year pilot-plant study on the biodegradability of TNT. A variety of conditions for degradation of TNT were investigated and best results were obtained with activated sludge microorganisms and supplemental nutrients. TNT was pumped continuously into an aerated oxidation ditch facility containing bacterial floc grown from activated sludge microorganisms supplemented with cornsteep liquor. When TNT was supplied at a load of 10-50 mg/l, 97% of the TNT was removed at a feed rate of 0.25 to 8 liters/minute. Transformation products were identified as the reduction derivatives of TNT. Biotransformation appears to occur by a step-wise bioreduction of the aromatic nitro groups to form amines which eventually react to form complex polar products. Studies with completely labeled ^{14}C-TNT demonstrated less than 0.4% $^{14}CO_2$ formation. The remainder of the ^{14}C-activity was equally distributed between the bacterial floc (solid) and the effluent (liquid) phase. No evidence of aromatic cleavage was observed. This study indicates that although TNT is toxic to many microorganisms at concentrations greater than 100 mg/l, it may be biologically transformed on a continual basis by a relatively simple process in the presence of supplemental nutrients.

The effect of environmental conditions such as sunlight, shade, darkness and added sediment on the fate of TNT in an aqueous environment has been studied by Burlinson (1980). Aerated river water was spiked with 20 mg/l TNT and incubated under the specified environmental conditions in cylindrical pyrex glass crocks. Each crock was monitored for disappearance of TNT, formation of photo- and biotransformation products, ATP, and biological activity. The disappearance of TNT and formation of its degradation products were followed by HPLC. To verify peaks observed by HPLC, samples were extracted, dried and concentrated for TLC separation. Mass spectrometry was used for positive identification of the compounds extracted from the TLC spots. ATP analyses and microbiological plate count determinations were performed on samples from each crock, daily for several days, then every other day. ATP determinations were not consistent with increases observed in microbial populations. The results of these experiments are presented graphically in Figure 4. The three crocks in sunlight demonstrated TNT concentrations approaching zero in 6-8 days, whereas over 30 days were needed for 90% TNT to disappear from the dark and shaded crocks. The main degradation product formed in the TNT river water subjected to direct sunlight was 1,3,5-trinitrobenzene (10%) with only a trace of 2-amino and 4-amino dinitrotoluene formed. Samples in the dark produced the known biotransformation products of TNT. These studies carefully monitored disappearance of TNT as well as formation and identification of transformation products. No evidence for ring cleavage was observed.

Parrish (1977) tested 190 pure cultures of fungi and reported that 183 strains were able to transform TNT at concentrations of 100 mg/l in a medium containing glucose and basal salts. Enzyme activity was found in mycelia and not in culture filtrates. Filtrates were analyzed and the transformation products found were the same as those identified by other investigators. Studies with ring-labeled ^{14}C-TNT gave no evidence for cleavage of the carbon skeleton.

SRI (1980) is currently studying the environmental fate of TNT. Using 100 mg/l ^{14}C-TNT solutions, they observed rapid transformation of the TNT in the presence of high concentrations of microorganisms. Biotransformation

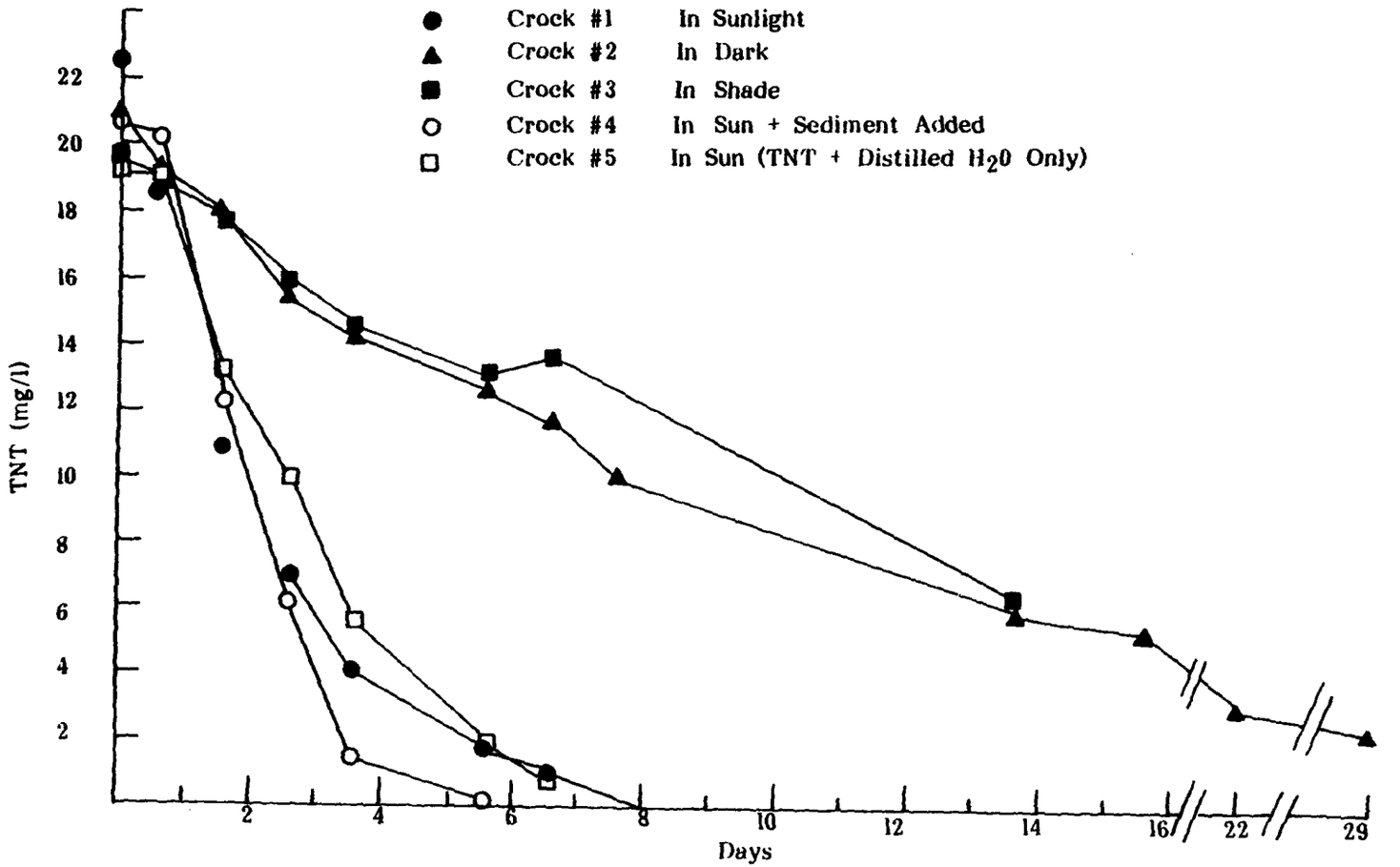


Figure 4. Disappearance of TNT in 'natural water' (Burlinson, 1980)

also proceeds more readily in the presence of sediment. The 2-amino and 4-aminodinitrotoluenes were identified as biotransformation products. These two isomers comprise about 30% of the ^{14}C-TNT and have an equal probability of being formed. Small amounts of the 2,4-diaminonitrotoluene were also found. The typical coupling products were also identified (see Figure 3). No evidence for ring cleavage was found.

b. Aerobic Microbial Degradation of TNT as the Sole Carbon Source

Several reports in the mid-1970's contained information which suggested that a number of microorganisms could utilize TNT as a sole carbon source. Won *et al.* (1974) reported that TNT enrichment cultures (*Pseudomonad*-like organisms) isolated from mud and water grew in a basal salts media containing TNT as the only source of carbon. Studies were carried out at 30-32°C with rotary agitation. For accelerated degradation, glucose or yeast extract were essential. Complete dissimilation of 80 mg/l TNT within 24 hours occurred in medium supplemented with 0.5% yeast extract. TNT was transformed to tetranitro azoxytoluene, monoamino dinitrotoluene, hydroxylamine dinitrotoluene and nitrodiaminotoluene.

Traxler (1974) also reported utilization of TNT as a sole source of carbon by Gram-negative bacteria isolated from various sources. Nineteen of fifty-seven isolates demonstrated increased turbidity upon inoculation into mineral salts basic media with TNT as the sole source of carbon. The addition of yeast extract (0.01%) stimulates microbial growth on TNT as shown in Figure 5. Some of the cultures tested were found to be a mixed culture which, when purified did not show an increase in turbidity with TNT as the sole source of carbon. The effect of temperature on the growth of the organism and disappearance of TNT was investigated with basal salts basic media containing 100 mg/l yeast extract and 100 mg/l TNT incubated at 25°, 30°, 35°, 40° and 45°C. Increases in turbidity were observed up to 40°C, although the percentage of TNT remaining did not differ significantly.

The relationship between initial TNT concentration in the medium and percent TNT utilization by isolate IIBX was inversely related to TNT concentration. This inverse relationship suggests TNT inhibition or inhibition by metabolic products. With a second isolate (I-2-5) obtained by mutation of an isolate from boiler plant effluent, a direct relationship between substrate concentration and percent of TNT utilized was observed. As shown in Figure 6, 62% of the TNT was removed from 100 mg/l TNT solution after 20 hours of incubation.

Experiments were conducted to determine the fate of TNT utilizing ring-UL-^{14}C-TNT (100 mg/l), basal salts media and 0.01% yeast extract. In the first two studies, 3.8% and 5.9% of the total radioactivity was recovered. A second set of studies resulted in 17.4% and 32.6% recovery of the total activity introduced into the system. Based on low levels of $^{14}CO_2$ evolved (0.02-0.26% of added ^{14}C-TNT), Traxler (1974) suggested that heterotrophic carbon dioxide fixation might be important in this system and that most of the metabolized carbon from the ^{14}C-TNT was used for the synthesis of cellular material. Studies with $NaH^{14}CO_3$

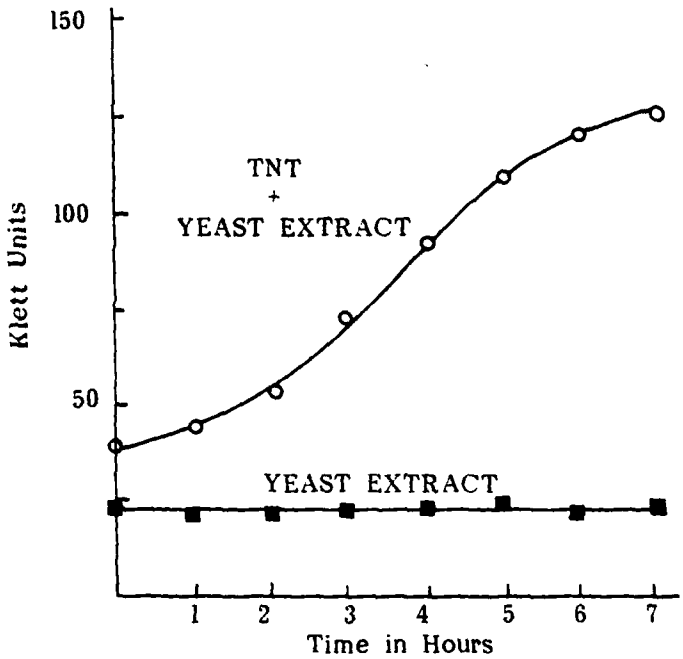


Figure 5. Growth of IIBX on TNT and yeast extract (Traxler, 1974)

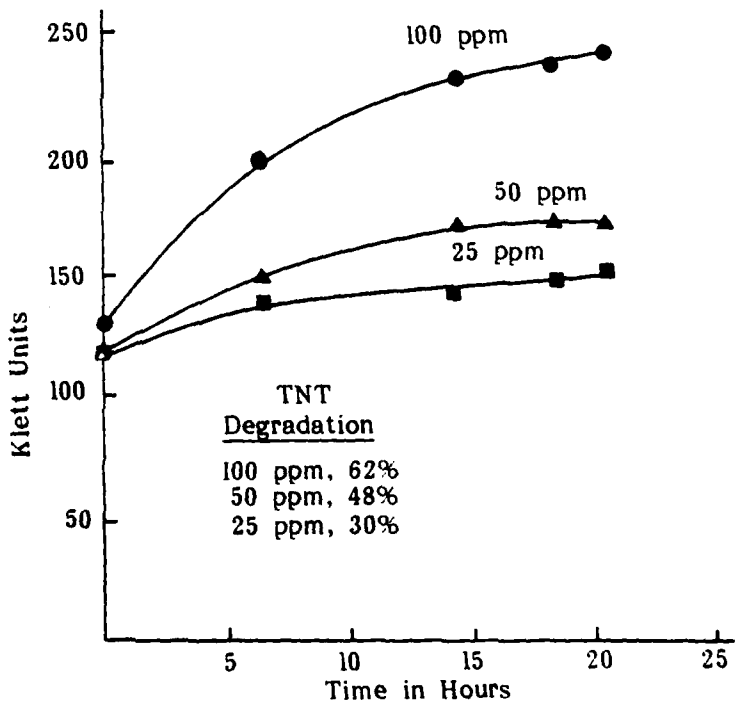


Figure 6. Effect of TNT concentration on growth response of I-2-5 and percent degradation. (Traxler, 1974)

demonstrated heterotrophic carbon dioxide fixation by one of the cultures growing on TNT. It was concluded that organisms may be capable of TNT ring cleavage and that the ^{14}C incorporated into cellular material was not in the form of nitroaromatic compounds. With high cell concentrations, TNT was effectively removed from the media with or without yeast extract or other organic nutrients. For example, 95% of the TNT was removed from media containing 100 mg/l in 18 hours with 14 mg/ml cells. At low cell concentrations, removal of TNT from the medium was poor.

Amerkhanova and Naumova (1978) studied bacterial utilization of TNT as the sole source of nitrogen and carbon for growth. Organisms used in the studies were an *E. coli* strain from the collection of the Faculty of Microbiology, University of Kazan and a *Ps. denitrificans* strain isolated from soil polluted by industrial effluent waters. Bacteria were cultured on a basic synthetic medium containing glucose, ammonium sulfate, magnesium sulfate, KH_2PO_4, and Na_2HPO_4. TNT was added at an amount equal to 0.02% to the basic synthtic medium without glucose. TNT was determined by a method based on its reaction with sodium sulfate in an alkaline medium. As illustrated in Figure 7, *E. coli* was less sensitive to the toxic action of TNT than *Ps. denitrificans*. The nitrogen in TNT was less accessible to *Ps. denitrificans* than to *E. coli*. The carbon in TNT was not utilized by *E. coli* and was accessible only to a very limited degree to *Ps. denitrificans*. Disappearance of TNT from the medium was enhanced when 0.5 g/l yeast extract was added. No isolation or identification of degradation products was reported in this study.

These studies were continued (Amerkhanova and Naumova, 1979) and the metabolic conversion of TNT by *Ps. denitrificans* was monitored. This microorganism converted TNT at a concentration of 200 mg/l by a series of reductions. The dynamics of TNT conversion are illustrated in Figure 8. Isolation and separation of the reduction products were performed by TLC. Metabolites with partially reduced nitro groups were detected with p-dimethylaminobenzaldehyde, nitroaryls with the same reagent after preliminary reduction with $SnCl_2$. Aromatic amino compounds were eluted from chromatograms and recrystallized from benzene. TNT was determined by two methods. The first method was based on reaction with sodium sulfite in alkaline medium and the second based on the action of alkalies on aromatic polynitro compounds which result in production of salts of acid forms which stain the solution yellow or orange. It is interesting that these investigators found evidence for preferential reduction of the nitro group in the 2-position whereas other investigators have found preferential reduction of the 4-nitro group. No evidence for ring cleavage was conclusively demonstrated.

Microorganisms which "grow" in a medium with TNT as a sole carbon source must degrade the TNT. To date increases in turbidity and the disappearance of TNT from the medium have been used as evidence of TNT metabolism by microorganisms. Increases in turbidity as a measure of growth should be confirmed by plate counts. Evolution of $^{14}CO_2$ from ring-labeled ^{14}C-TNT has been insignificant (0.02-0.26%). Reports of increased oxygen uptake (Chambers *et al.*, 1963) in the presence of TNT may be the result of experimental variation, i.e. some experiments demonstrate more oxygen uptake than others (Klausmeier, 1975). Subsequent studies have found no evidence of biological ring cleavage and other investigators (SRI, 1980) have not observed the utilization of TNT as a sole carbon source by mixed microbial populations.

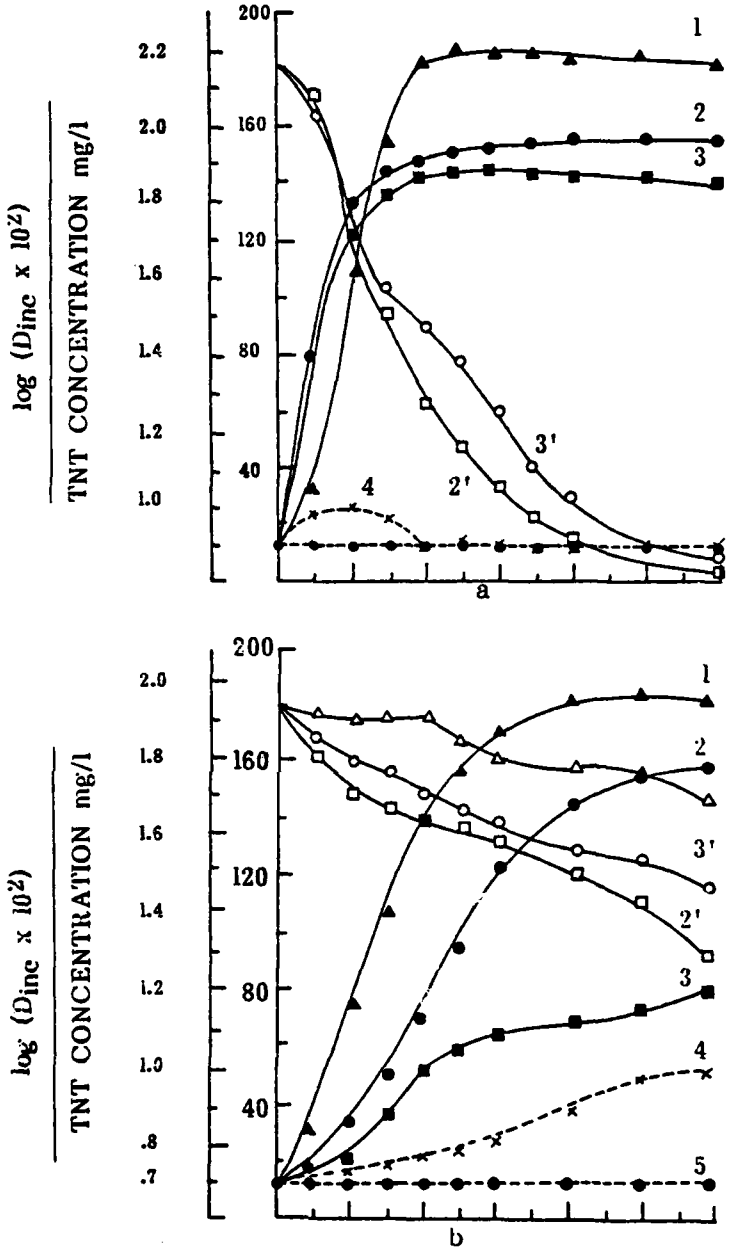


Figure 7. Utilization by bacteria of TNT as a source of nitrogen and carbon nutrition. (Amerkhanova and Naumova, 1978) a) *E. coli*; b) *Ps. denitrificans*; 1) growth on synthetic medium; 2) growth on synthetic medium with TNT; 3) TNT as a nitrogen source; 4) TNT as carbon source; 5) mineral background; 2',3') decrease in TNT concentration in corresponding test variants.

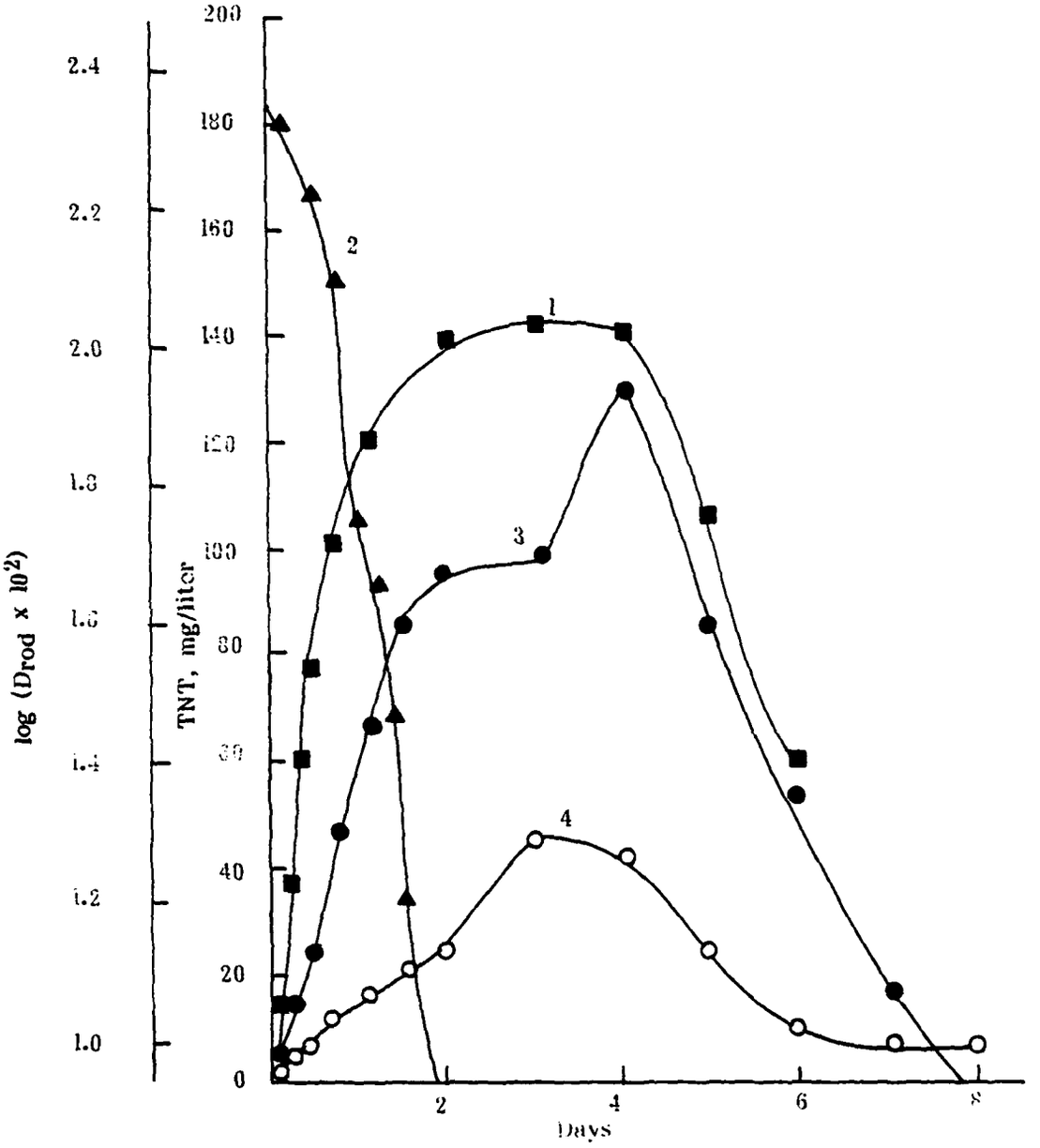


Figure 8. Dynamics of the conversion of TNT and the formation of amino derivatives during growth of *Ps. denitrificans* (Amerkhanova and Naumova, 1979): 1) growth; 2) TNT; 3) 2-amino-4,6-dinitrotoluene; 4) 4-amino-2,6-dinitrotoluene.

c. Anaerobic Microbial Degradation of TNT

A number of studies have investigated the biodegradation of TNT in an anaerobic environment. Klausmeier (1975) investigated the effects of anaerobic bacteria on the transformation of TNT. Complete reduction of the three nitro groups to 2,4,6-triaminotoluene was achieved. Aerobic and anaerobic enrichment cultures were used to test some of the *Pseudomonad* isolates; *Clostridium* sp., *Veillonella* sp. and E. coli were also studied. A variety of reduced compounds was found but there was no evidence of ring cleavage. McCormick et al. (1976) used an enzyme preparation from *Veillonella alkalescens* to test a variety of nitroaromatic compounds for reduction by hydrogen to form corresponding arylamines. They demonstrated that TNT could be transformed by anaerobic microorganisms as well as by aerobic organisms. Continuation of this work (McCormick et al., 1978) indicated that under aerobic conditions, the hydroxylamine-dinitrotoluene undergoes oxidative coupling to form azoxy compounds. Earlier Channon et al. (1944) stated that azoxy compounds may be formed by a coupling reaction of corresponding hydroxylamines and were not the direct metabolic products of TNT. McCormick et al. (1978) found that under semi-anaerobic conditions, most of the hydroxylamino species are enzymatically reduced to the amine. Jerger and Chynoweth (1966) reported anaerobic microbial transformation of TNT in anaerobic digestors. They observed partial disappearance of TNT and the appearance of transformation products, one of which was identified as 4-hydroxylamino-2,6-DNT. SRI (1980) also monitored transformation of TNT under anaerobic conditions and observed reactions and products similar to those found in aerobic studies.

d. Microbial Degradation of TNT in Soils

TNT behavior in soil has also been studied. Investigators at the Natick Research and Development Command extracted soil samples from minefill operations at Crane NAD for TNT analysis (Natick, Quarterly reports 1975-1978). Some of the compounds identified were TNT, RDX, long chain aliphatic compounds and other nitro aromatic compounds. Acetone extracts from composted TNT were analyzed by thin layer chromatography and high pressure liquid chr matography and indicated no residual TNT. NMR and IR analysis indicated that small amounts of aromatic material were present. Thin layer chromatography indicated the presence of at least four compounds, one of which was tentatively identified as 1,3-dinitrobenzene.

Osmon and Andrews (1978) studied degradation of TNT in soil which had been amended with supplemental nutrients. Higher conversion rates were observed when soil was maintained at 40% moisture than at 20% moisture and the periodic re-addition of nutrient maximized the conversion rate. Bioconversion at higher concentrations of TNT was relatively slow. End products included 4-amino-dinitrotoluene indicating that TNT was not completely mineralized in the soil system. The production of undesirable end products and the slow rate of degradation render this method impractical as a means of disposing of large quantities of TNT.

Hoffsommer (1980) noted that TNT sorbed to charcoal was transformed into trinitrobenzene and a number of other uncharacterized compounds. He suggested that TNT added to soil may be converted into a variety of products.

e. Microbial Degradation of TNT in Composts

The use of composting as a method for degrading TNT has been investigated (Osmon and Andrews, 1978). The composting process utilizes a combination of heat and thermophilic bacteria to affect degradation of organics. These researchers have shown successful composts with TNT comprising as much as 10% of the dry weight of the original material. The composting process was optimized by shredding or grinding the initial materials into small particles, maintaining proper moisture and aeration levels and a proper nutrient balance, i.e. carbon to nitrogen ratio.

The effectiveness of the composts with varying initial TNT concentrations is shown in Table I. Only small amounts of TNT were recovered from the composted material. As shown in Table II, no indication of the known bioconversion products, i.e. 2-amino and 4-aminodinitrotoluene and azoxy compounds, was obtained by the TLC and GC-MS. When the composting process was monitored using ^{14}C-TNT, only small amounts of ^{14}C-TNT were converted to $^{14}CO_2$ or cell mass. The bulk of the material was found to be water soluble and not solvent extractable. Analysis of the transformation products by the Navy has not been completed. The Natick Laboratory will attempt to chemically analyze the resulting compost material.

TABLE I. TNT DEGRADATION IN COMPOSTS
(Osmon and Andrews, 1978)

Days	(% TNT in Compost)			
0	1.0	5.0	5.0	10.0
2	0.9	4.3	-	-
4	0.4	3.0	-	-
6	-	-	2.8	2.0
7	0.4	3.3	-	-
9	-	-	3.1	5.5
11	0.2	2.5	-	-
13	-	-	1.6	3.7
17	-	-	1.4	3.8
18	0.13	2.2	-	-
32	0.05	1.0	-	-
38	-	-	0.8	2.0
46	0.01	0.5	-	-
52	-	-	0.7	1.2
53	0.01	0.4	-	-

TABLE II. COMPOUNDS NOT DETECTED AS PRODUCTS OF TNT COMPOSTING (Osmon and Andrews, 1978)

2,5-dinitrotoluene

3,5-dinitrotoluene

2,4-dinitrotoluene

2,4,6-trinitroethylbenzene

2,6-dinitrotoluene

2,4,6-trinitrobenzaldehyde

2,4,6-trinitrobenzyl alcohol

2,4,6-trinitrobenzoic acid

2,4,6-trinitrophenol

4-amino-2,6-dinitrotoluene

2,4-diamino-6-nitrotoluene

4-hydroxylamine-2,6-dinitrotoluene

2,2',6,6'-tetranitro-4,4'-azoxytoluene

4,4',6,6'-tetranitro-2,2'-azoxytoluene

all isomers of dinitrophenols

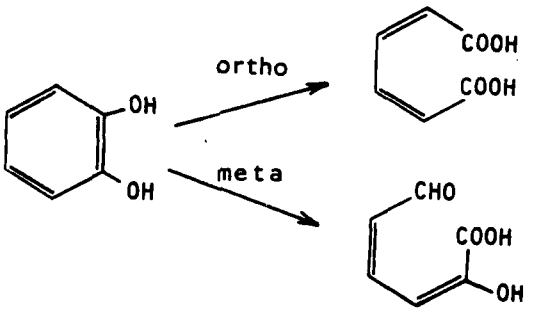
III. SUMMARY AND EVALUATION OF LITERATURE

Many studies have been conducted to determine the biodegradability of RDX, TNT and DNT. All of these studies have utilized adapted pure cultures or adapted mixed cultures of microorganisms. No work has been performed using mutant or genetically engineered microbes for degradation of RDX, TNT or DNT.

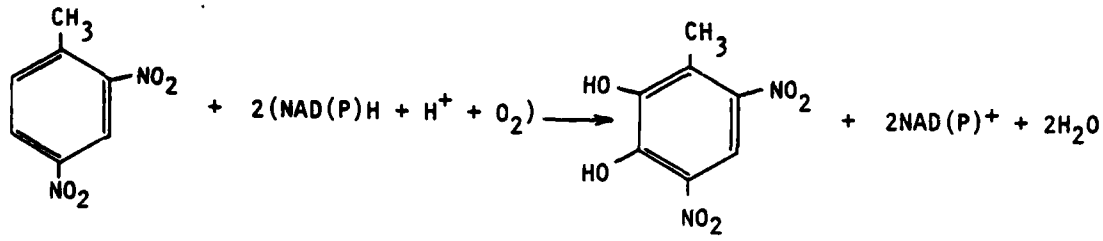
A. Dinitrotoluene

Evaluation of the literature indicates that 2,4-DNT is transformed into amino and azoxy compounds by pure cultures and cell extracts. Complete degradation of the molecule does not occur under these conditions. With high concentration of mixed microbial populations, complete mineralization of 2,4-DNT occurs within one week. The 2,6-DNT isomer is not degraded under these conditions. Although not conclusive, the evidence indicates that co-metabolism by two or more species is necessary for mineralization of 2,4-DNT.

For ring cleavage to occur, the benzene nucleus must carry two hydroxyl groups in the ortho or para positions to each other. If the hydroxyl groups are not on the ring, they must be inserted for ring fission to occur. Monooxygenases are responsible for hydroxylation of aromatic rings by bacterial systems. The monooxygenases are usually flavoproteins that require reduced pyridine nucleotide for their activity. These enzymes are usually very substrate specific. After hydroxylation has occurred, dioxygenase cleaves the ring by simultaneous insertion of two atoms of molecular oxygen. With ortho hydroxyl groups, ortho or meta cleavage can occur:



The question then remains as to why 2,4-DNT is degraded and 2,6-DNT is not. One potential explanation for the difference in biological degradability is as follows. The biodegradation of toluene has been extensively studied. This chemical has been shown to degrade via the 3-methylcatechol intermediate. If the monooxygenase enzyme is truly specific, then DNT must also proceed through the 3-methylcatechol intermediate. For 2,4-DNT, this intermediate is possible.



2,4 DNT

2,4-dinitro-5,6-dihydroxytoluene

However, with 2,6-DNT the formation of this intermediate is not possible.

B. 2,4,6-Trinitrotoluene

Many organisms tolerate moderate levels of TNT in aqueous solutions and are effective in transforming TNT at low concentrations (less than 100 mg/l). The addition of nutrients and/or sediment significantly enhances the rate at which TNT is biotransformed. Most organisms (bacteria, fungi, rats, man) appear to produce similar transformation products from TNT. The 2-amino and 4-aminodinitrotoluenes and condensation products account for approximately 99% of the TNT transformed in natural systems. The ortho or para dihydroxy compounds, which normally precede ring cleavage, can not be formed from the molecule under ordinary conditions. (No evidence has been found for ring cleavage.)

TNT breakdown does not appear to occur to any appreciable extent in unenriched soil. Addition of nutrients encourages microbial activity and may result in a very slow rate of TNT transformation. TNT may react with soil to form dozens of compounds which may or may not be persistent. A number of these products may be strongly sorbed onto the soil and may not be available for further degradation.

Composting appears to be the technique with the greatest potential for successful TNT degradation. In this system, concentrations of TNT up to 10% of dry weight do not significantly inhibit the composting process. Although rapid transformation of TNT occurs, very little TNT is converted to CO_2 or cellular material during the process; the bulk of carbon is water soluble and not solvent extractable. Further analysis of the composting products of TNT are needed to insure that the process does not produce unwanted toxic or persistent compounds.

C. RDX

RDX degradation proceeds very slowly under aerobic conditions. However, anaerobic degradation proceeds rapidly with the formation of several products which have been tentatively identified as formaldehyde, trinitrosotriazine, hydrazine and symmetric and unsymmetrical dimethylhydrazine. Yeast extract appears to increase the rate of anaerobic degradation.

IV. USE OF MUTANT OR GENETICALLY ENGINEERED MICROBES FOR DEGRADATION OF TNT, DNT AND RDX

As pointed out in Section III, the use of mutant or genetically engineered microorganisms for degrading DNT, TNT or RDX was not reported in the literature. Several researchers in the area of mutant and genetically engineered microorganisms were contacted to determine if organisms had been developed that would degrade TNT, DNT or RDX. The information obtained from these contacts is summarized below.

Dr. Ananda M. Chakrabarty, a former General Electric microbiologist, created the oil-eating bacteria that was the subject of a landmark Supreme Court ruling in June, 1980. Dr. Chakrabarty is now directing a team of scientists at the University of Illinois trying to develop microorganisms to eat toxic wastes. Chemicals for which microbes are being developed are high volume toxics such as PCBs (polychlorinated biphenyls), chlorobenzenes and dioxins found in herbicides such as Agent Orange.

General Electric, the company under which the oil-eating bacterium was created, has no plans to commercialize the oil bug. They believe that the market is not large enough to warrant the cost involved in production of the microbes since other less expensive techniques are available for removing oil from the environment. According to Bill Austin, General Electric is not pursuing research in this area and has no current plans to do so in the future.

Battelle Columbus Laboratories are studying the predilection of certain strains of *Pseudomonas* to eat such compounds as the defoliant 2,4-D. The Battelle team have identified plasmids as the site for genes encoding the traits necessary to degrade 2,4-D. Genetic engineering techniques are being employed to remove these trait-specific genes and put them in *Pseudomonas* species that otherwise do not degrade 2,4-D. These researchers plan to attempt gene insertion into bacteria other than *Pseudomonas* to map the range of life that might be programmed by gene exchange to devour unwanted chemical wastes such as polychlorinated biphenyls and kepone (West, 1980).

Genex, a genetic engineering firm in Rockville, Maryland, is working in cooperation with one client in screening naturally occurring microorganisms for degradation of a particular chemical. When the desired microorganism(s) are obtained, genetic adaptation will be attempted to enhance the rate at which microbial degradation occurs. Dr. Jackson states that Genex has no particular expertise in the area of biodegradation although the company has considerable expertise in the field of genetic engineering. Future plans include increased participation in the area of adapted/mutant microorganisms for biodegradation.

Genentech, San Franciso, California, is also a genetic engineering firm. At the current time, Gary Hooper states that Genentech is not involved in the field of biodegradation. However, in a meeting early in September, the subject was discussed and it appears that Genentech will enter the field of biodegradation by adapted/mutant microorganisms in the near future.

Polybac is a Pennsylvania corporation which specializes in biomass engineering. In cooperation with clients or as a service to clients, microorganisms are screened for capability to degrade a specific compound or class of compounds. Adaptation of these microorganisms is undertaken and may be followed by mutagenic treatment to enhance the rate of microbial degradation of the selected compound(s). Polybac clients are mainly chemical companies and refineries. Selected microorganisms are often added to the waste treatment facilities at these chemical companies or refineries to get rid of toxic substances. Reports of industrial use of Polybac products include biological treatment of the phenoxy herbicides, 2,4-D and 2,4,5-T in a closed pure oxygen activated sludge treatment facility using biomass containing a strain of microorganisms (PHENOBAC) (Wachinski *et al.*, 1974). The use of PHENOBAC to enhance the operation of a refinery biological system (EXXON) was studied in a series of long-term controlled tests and results indicated that PHENOBAC was useful not only for start-up and improvement of routine operations, but for aiding in upset recovery as well (Tracy and Zitrides, 1979). NITROBAC (for removal of ammonia from wastewater) and PHENOBAC (to degrade heavy aromatics, saturated and unsaturated organics, phenolic compounds and cyanides) have been successful in treating wastes from the J.T. Baker Chemical Company (Blanchfield, 1979). PHENOBAC has also been used to clean up an ortho chlorophenol spill in Sturgeon, Mo. The lagoon in which the hazardous material plus contaminated runoff water were collected contained in excess of 600 ppm ortho chlorophenol. After one month of treatment with PHENOBAC, the ortho chlorophenol level had decreased to 25 ppm (Voras, 1980).

Polybac offers the following microbial products:

- PHENOBAC - substrate hydrocarbons in fresh water
- LIGNOBAC - substrate pulp and paper wastes
- PETROBAC - substrate hydrocarbons in salt water
- HYDROBAC - substrate hydrocarbons from refineries
- NITROBAC - substrate ammonia

Additional mutant/adapted organisms are available for degradation of halophenols, crude oil, cyanides, detergents, aliphatic amines, aryl amines and aryl halides. Polybac contact, Curtis McDowell, reports limited success with degradation of aniline, however, none of the Polybac current biological strains appeared to have a potential for degradation of cyclic nitramine or nitroaromatic compounds. Mr. Dowell would not clarify the statement he made concerning "some success with other nitro compounds." The company would be pleased to work with a client on selection of organisms for possible adaptation and mutation for degradation of the compounds of interest.

V. APPLICABILITY OF ADAPTED/MUTANT MICROORGANISMS TO INSTALLATION RESTORATION

A. Applicability of Adapted/Mutant Biodegradation for Removing TNT, RDX and DNT from Soils

The use of a mixed microbial population for degradation of low levels of DNT in soil appears to be a viable process. Degradation should be achievable by spraying the soil with an aqueous solution containing approximately 1% yeast extract and a high concentration of microorganisms. However, it is highly probable that any 2,4-DNT in the soil has been degraded over the years and no additional treatment is necessary.

Biological degradation of RDX appears most promising under anaerobic conditions in the presence of mixed microbial populations which have been adapted to the presence of RDX at low concentrations. RDX contamination in soils is not expected to exceed concentrations tolerated by microorganisms under experimental conditions (20 ppm), however, creating and maintaining anaerobic conditions for large quantities of soil is difficult. Large areas of land may be flooded to create anaerobic conditions and adapted microbial cultures could be seeded into the aqueous environment in conjunction with the appropriate nutrients. Alternatively, the contaminated soil could be excavated and then placed in an existing pond or lagoon (or in a newly created pond) for addition of the appropriate nutrients and adapted microbial populations.

Genetic engineering or mutation to produce aerobic organisms which could degrade RDX in soils is potentially possible. No mutant or engineered microorganisms which aerobically degrade RDX are currently commercially available. Sikka *et al.* (1980) indicate some degradation with the release of $^{14}CO_2$ takes place presumably under adapted aerobic conditions. These organisms could possibly be mutated or engineered to more efficiently degrade RDX in soils. Genetic engineering or mutation of microbes for aerobic degradation of RDX would be a costly undertaking. It also has a high probability of failure. However, if organisms can be developed which degrade low levels of RDX in soil, the research cost may be more than offset by lower decontamination costs for facilities and routine wastewater treatment.

Complete biodegradation of TNT with adapted, mutant or genetically engineered mesophilic microorganisms is probably not achievable. However, the transformation products are normally complex polymers which may or may not be environmentally acceptable. Further toxicity studies on these transformation products are necessary to determine if they are hazardous and if biotransformation is an acceptable method for decontamination of soils containing low levels of TNT.

B. Applicability of Adapted/Mutant Biodegradation for Removing TNT, RDX and DNT from Sediments

The sediments in the bottom of the wastewater lagoons contain large amounts of explosives. The Army desires to decontaminate the sediment in these lagoons by the most expeditous and cost-effective methods available. Therefore, to compare the use of adapted/mutant biodegradation to other decontamination techniques, a standard lagoon has been adopted. The specific parameters of this lagoon are listed in Table III. For sizing the treatment process, it is assumed that there are 11 standard lagoons per site and all the the sediments will be decontaminated within one year or approximately one lagoon will be treated per month.

The applicability of adapted/mutant biodegradation to the decontamination of lagoon sediments must be based on the technical and cost feasibility of the process. From the available data, it appears that RDX and DNT will be degraded in a system containing an anaerobic step followed by an aerobic step. TNT will be biotransformed, but not degraded. The question as to whether RDX can be effectively treated aerobically is unanswered. However, for cost analysis purposes, we have assumed that RDX, DNT and TNT can be completely broken down by microbes in an aerated activated sludge biosystem. It has also been assumed that complete degradation of 100 mg/l TNT and 30 mg/l RDX, and the DNT to less than 0.1 mg/l will occur in three days. Thus a holding time of 30 days in the system would be required. Since the system must treat 5,477,000 to 14,940,000 l/day (1.477 to 3.947 mgd) capacity of the aeration tanks must be 16,431,000 to 44,818,000 liters (4.431 to 11.841 million gallons).

The degradation of these explosives could be carried out in a typical activated sludge system. This system would consist of the following processes:

- explosives solubilization tanks
- solids separation
- aeration tank
- secondary clarifier
- digester

The capital cost of building an activated sludge plant to handle these volumes of water is $4-6 million (based on costs for building the activated sludge treatment plant at Holston AAP [The Ralph M. Parsons Co., 1980]). Operating costs are estimated at approximately $1.53 million per year including labor ($100,000), power (90,000), nutrients and chemicals ($450,000) and water ($90,000). Thus, the minimum cost for clean-up of the 11 lagoons would be $6.53 - 7.53 million.

If time can be sacrificed for price, an alternative adapted/mutant biodegradation approach would be to use the lagoons as bioponds. The degradation time for an aerated lagoon will be significantly longer than in an activated sludge plant. From the available data, 7 days appears to be a reasonable time.

TABLE III. SCENARIO FOR TYPICAL LAGOON

Size	30.48 m x 45.72 m x 2.44 m deep (100 ft x 150 ft x 8 ft deep)
Depth of contaminated sediment	0.3 m (1 ft)
Volume of contaminated sediment to be treated	2617 m^3 (15,800 ft^3)
Weight of contaminated sediment to be treated	472,368-537,120 kg (1,084,512 - 1,183,104 lb*)
Lb of TNT to be treated	9847 - 26,856 kg (21,690 - 59,155 lb**)
Lb of RDX to be treated	4924 - 13,428 kg (10,845 - 29,578 lb**)
Lb of DNT to be treated	10 - 26.8 kg** (22 - 59 lb)
Lb of Tetryl to be treated	10 - 26.8 kg** (22 - 59 lb)
Total volume of water to be treated	164,337,000 - 448,204,000 liters (43.418.000 - 118,416,000 gallons)+
Water to be processed per day for treatment in 30 days	5,477,000 - 14,940,000 liters/day (1,447,000 - 3,947,000 gallons/day)

* Based on a sediment containing 20-50% solids having a density of 1.1-1.29 g/cm^3

** Based on the following explosive concentrations in dry sediment:

- 10% TNT, 5% RDX, 100 μg/g DNT, 100 μg/g tetryl

\+ Based on dissolving TNT to a concentration of 100 mg/l and RDX to 30 mg/l and RDX as the limiting case for solubility.

For this approach, the only capital equipment required would be a dredge to periodically loosen the sediment and aerator mixers to maintain the explosives in solution and provide dissolved oxygen for the microorganisms. Total capital cost for this type of decontamination would be:

1 dredger 300 gal/min	$ 50,000
11 lighter aerator-mixer $22,000/ea	242,000
installation	24,000
	$ 316,000

Operating costs would also be less than an activated sludge plant per year due to lower labor and maintenance requirements. Total yearly operating costs would be $760,000 including labor ($200,000), power ($100,000), nutrients and chemicals ($450,000) and water ($10,000). Total time required to decontaminate the eleven lagoons simultaneously will range from 1.06 to 2.9 years. Therefore, the total cost will range from $1,122,000 - $2,520,000.

Adapted/mutant biological treatment using the lagoons as treatment ponds may appear to be a cost effective alternative for decontamination of lagoon sediment. However, the technical uncertainties are very great. It is not known if the organism can survive in contact with the high levels of explosives present in the lagoons even though the solubility of the explosives in the aqueous phase is limited. The feasibility of engineering an aerobic mutant to degrade RDX is unknown. If anaerobic processes are required to degrade RDX followed by aerobic treatments to degrade the other chemicals, the costs of treatment will increase significantly. Finding or engineering a microbe to degrade TNT is an even bigger uncertainty. Genetic engineering and/or chemical manipulation of the wastes before biotreatment can be tried, however, the probability of success is very low. Based on these observations, the use of adapted/mutant biodegradation for removing TNT, RDX and DNT from lagoon sediment is not recommended.

C. *Applicability of Composting for Removal of TNT, RDX and DNT from Contaminated Lagoon Sediment*

The available evidence indicates that composting can effectively degrade TNT in concentrations up to 10% of the compost pile. Although the final products have not been identified, they are water soluble and probably non-toxic. Biodegradation of RDX and DNT in a compost system has not been demonstrated. However, if the more chemically stable TNT is degraded, the mixed microbial populations present should degrade the DNT and the thermophiles under semi-anaerobic conditions may degrade RDX. Further compost studies are needed to evaluate this system with respect to degradation of the explosives and the resultant by-products.

A compost operation such as described by Osmon and Andrews (1978) involves preparation and mixing of the compost material, and the composting system itself. The preparation and mixing operation should consist of a receiving area for domestic refuse, separation of the non-compostable items and pulverization of the refuse material to small particle size. The compost material and lagoon sediment would then be mixed in the lagoon. For this operation, the major portion of the water should be predrained from the lagoon. The wet sediment would be dredged and mixed with the compost material. The mixed material would be placed in discreet piles in the lagoons.

During the composting, the pile must be mixed and turned frequently to maintain a high oxygen level and to promote an infusion of outer undegraded material into the active interior of the compost mass. Additional nutrients may also be added to maintain a C/N ratio of 30:1. Runoff water is recirculated to maintain a moisture level of 40-60%. Temperature, pH, moisture levels and products in the water runoff from the pile should be monitored daily.

The capital and operating costs for decontamination of the 11 standard lagoons is presented in Table IV. It is anticipated that 2-4 ninety-day composing operations will be necessary to completely decontaminate each lagoon. All 11 lagoons can be decontaminated simultaneously, thus, the entire operation can be completed within one year. Total decontamination cost should not exceed $686,577 and may be as little as $505,794. Thus, composting appears to be a very cost effective approach for decontamination of lagoon sediments. However, the technical feasibility of using this technique to degrade high concentrations of TNT and RDX in lagoon sediments has not been fully demonstrated.

TABLE IV. CAPITAL AND OPERATING COSTS FOR COMPOSTING OF CONTAMINATED LAGOON SEDIMENTS

Capital Costs		1980*
Receiving hopper		$ 13,915
Apron conveyor 1.22 m x 14.24 m		54,450
Belt conveyor 1.22 m x 16.76 m		19,360
Picking station		6,050
Hammermill 149.2 kilowats (200 h.p.)		52,030
Magnetic separator		6,655
Paddle mixer		24,200
Compost turner		42,350
Recirculating pumps (11)		88,000
Dredge 1136 liters/min (2.44 m x 6.1 m)		50,000
		$ 307,010
Operating Costs		
Plant manager/technician	(1)	48,702
Technician	(1)	39,930
Operator	(3)	165,700
Chemist	(1/2)	30,250
Microbiologist/program manager	(1/2)	27,225
Repair and maintenance		6,050
Utilities		67,710
		$ 379,567

*Costs based on data by Osmon and Andrews (1978) modified to fit lagoon scenario.

VI. CONCLUSIONS

The available data on the microbial degradation of TNT, 2,4-DNT and RDX have been evaluated to determine if the use of adapted/mutant microorganisms should be further studied as a means of removing these compounds from the environment.

Adapted/mutant biological treatment could be useful in degrading low levels of RDX and DNT in soils. However, TNT would be transformed but probably not degraded by adapted/mutant microorganisms. The cost-effectiveness of this type of treatment is questionable. The DNT is probably degraded in the environment without treatment. RDX degrades rapidly under anaerobic conditions, but creating anaerobic conditions in large soil masses is difficult and expensive. Attempting genetic engineering with anaerobic organisms, would be very costly and time consuming with no guarantee of success. There is no evidence in this literature that TNT can be degraded by mesophilic microorganisms. Because no microorganisms have been isolated which can utilize TNT as a sole carbon source (cleavage of the ring), genetic engineering aimed at TNT biodegradation does not appear to be feasible. Based on the available data, the potential for success with adapted/mutant microorganisms for degradation of explosives in the soil and sediment does not appear to justify the expense and time required for genetic engineering.

Composting appears to be the most promising method for effective, economic, ecologically acceptable disposal of TNT from lagoon sediments. This method should also be effective for disposal of DNT based on structural similarity and because DNT is degraded aerobically by mixed microbial populations in an aqueous system. It is highly probably that this technique is also applicable to the disposal of RDX.

VII. RECOMMENDATIONS

Based on the review and evaluation of the available data on biological degradation of TNT, DNT and RDX in soil or sediment, further work with adapted/mutant microorganisms for this purpose is not recommended. Since composting appears to be the method of choice for acceptable disposal of TNT, furher evaluation of the Navy's results should be undertaken. This evaluation should include a detailed study of the analytical methodology used to determine TNT degradation and identification of breakdown products. If after this evaluation, composting appears to be a viable process, a series of bench-scale composting studies to determine the usefulness of the process for decontamination of the three explosives in lagoon sediments should be undertaken. A proposed scheme for these studies is presented below:

1. Initiation of bench-scale composting studies to determine the applicability of the composting technique to disposal of RDX, DNT and TNT utilizing individual studies with ^{14}C-RDX, ^{14}C-TNT and ^{14}C-DNT.

   - Utilization of two concentrations for each explosive to be studied with concentration selection based on data obtained during the literature review and preliminary ARC analyses of the sediments

   - Monitoring of each study for degradation of the ^{14}C-labelled compounds, appearance of degradation products and identification of degradation products when possible.

2. Initiation of bench-scale composting studies to determine the applicability of the technique for disposal of mixtures of TNT, DNT and RDX.

   - Utilization of at least three different explosives ratios based on data obtained in the study above.

   - Monitoring for degradation of the explosives, appearances of degradation products and identification of degradation products when possible.

3. Initiation of bench-scale composting studies utilizing lagoon sediment contaminated with RDX, TNT and/or DNT.

   - Monitoring for degradation of the explosives, appearance of degradation products and identification of degradation products when possible.

4. Detailed economic analysis for decontamination of selected lagoons.

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APPENDIX

Literature Search

The search strategy utilized to find information on the effectiveness of adapted/mutant biological strains to treat soil and lagoon sediment contaminated with explosives was as follows:

Key Words:
- TNT
- Trinitrotoluene
- DNT
- Dinitrotoluene
- RDX
- Cyclotrimethylene trinitramine
- Biodegradation
- Decontamination
- Degradation
- Land reclamation
- Land restoration
- Sludge
- Microorganisms
- Bacteria
- Fungi
- Inducible enzymes

Strategy:

Key words in Set 1 were combined in an "or" mode.

Key words in Set 2 were combined in an "or" mode.

Set 1 and 2 were combined in an "and" mode.

An expanded literature search was subsequently initiated and completed. Additional keywords used in the search included mutant microorganisms, organic treatment, treatment, pollutant treatment, pollution and genetic engineering.

Files searched included the following:

- File 6 - NTIS
- File 10 - AGRICOLA
- File 40 - ENVIROLINE
- File 76 - IRL LIFE SCIENCES COLLECTION
- File 50 - CAB ABS
- File 2 - CA SEARCH

- File 3 - CA SEARCH

- File 4 - CA SEARCH

Additional pertinent references were obtained from a hand search of journals and references from pertinent papers.

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LIST OF SYMBOLS AND ABBREVIATIONS

mg	-	milligram
l	-	liter
RDX	-	hexahydro-1,3,5-trinitro-1,3,5-triazine
nm	-	nanometer
GC	-	gas chromatograph
HMX	-	cyclotetramethylenetetranitramine
ppt	-	parts per thousand
μg	-	microgram
ml	-	milliliter
M.P.	-	melting point
TLC	-	thin layer chromatography
MS	-	mass spectrophotometer
IR	-	infrared spectrophotometer
HPLC	-	high pressure liquid chromatograph
DNT	-	2,4-dinitrotoluene
^{o}C	-	temperature in degrees centigrade
g	-	gram
μl	-	microliter
UV	-	ultraviolet
M^{-1} cm^{-1}	-	per mole per centimeter
ATP	-	adenosine triphosphate
TNT	-	2,4,6-trinitrotoluene
NMR	-	nuclear magnetic resonance spectrometer
ppm	-	parts per million
pH	-	hydrogen ion concentration
sp.	-	species
SRI	-	Stanford Research Institute
K_p	-	partition coefficient


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